

Bacterial Antisense RNAs: How Many Are There, and What Are They Doing?*

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Key Words

small RNA, gene regulation, transcription interference, mRNA
stability

Abstract

Antisense RNAs encoded on the DNA strand opposite another gene have the potential to form extensive base-pairing interactions with the corresponding sense RNA. Unlike other smaller regulatory RNAs in bacteria, antisense RNAs range in size from tens to thousands of nucleotides. The numbers of antisense RNAs reported for different bacteria vary extensively, but hundreds have been suggested in some species. If all of these reported antisense RNAs are expressed at levels sufficient to regulate the genes encoded opposite them, antisense RNAs could significantly impact gene expression in bacteria. Here, we review the evidence for these RNA regulators and describe what is known about the functions and mechanisms of action for some of these RNAs. Important considerations for future research as well as potential applications are also discussed.

sRNA: small, regulatory RNA

Target RNA: RNA regulated by base pairing with a *cis*- or *trans*-encoded RNA

***trans*-encoded sRNA:** small, regulatory RNA encoded at a genomic location distinct from the target RNA

Hfq: RNA chaperone protein required for base pairing by many *trans*-encoded sRNAs

***cis*-encoded RNA:** regulatory RNA encoded on the DNA strand opposite its target mRNA; also termed antisense RNA

UTR: untranslated region

INTRODUCTION

In the past ten years, there has been an explosion in the identification of small, regulatory RNAs (sRNAs) encoded on bacterial chromosomes. Although some of these regulatory RNAs act by binding to and modulating protein activity, the majority of characterized sRNAs act by base pairing with target mRNAs. These base-pairing sRNAs fall into two categories: *trans*-encoded and *cis*-encoded. The *trans*-encoded sRNAs are encoded at genomic locations distinct from the mRNAs they regulate and thus generally only share limited complementarity with their targets. Due in part to the ability to act via limited complementarity, many of these *trans*-encoded sRNAs have multiple mRNA targets. In a number of bacteria, this type of base pairing requires the RNA chaperone protein Hfq. Thus far, the *trans*-encoded sRNAs are the most extensively characterized sRNAs and are discussed in a number of recent reviews (72, 119). In general, there has been less focus on *cis*-encoded sRNAs. These RNAs are transcribed from the DNA strand opposite another gene on bacterial chromosomes and thus have perfect complementarity to this target. As we describe here, increasing numbers of bacterial *cis*-encoded RNAs of various sizes, which we denote as antisense RNAs, are being reported and many are being characterized, raising questions about their physiological roles and mechanisms of action.

Ironically, antisense RNAs encoded on plasmids, phages, and transposons were among the first regulatory sRNAs to be studied. In 1981, Tomizawa and colleagues showed that the 108 nucleotide RNAI RNA controls the copy number of plasmid ColE1 by preventing RNAII processing to generate replication primers (106, 107). That same year, Nordström and colleagues identified the approximately 90 nucleotide CopA RNA, which controls the copy number of plasmid R1 by regulating the translation of the RepA replication initiator protein (100). A few years later, the 70 nucleotide RNA-OUT of the transposon Tn10 was found to affect transposition by repressing transposase synthesis (96). In addition, the

69 nucleotide Sar RNA of bacteriophage P22 (55, 120) and the 77 nucleotide OOP RNA of bacteriophage λ (51) were reported to repress synthesis of the Ant and cII phage proteins, respectively. Another type of plasmid antisense RNA discovered early on was the approximately 70 nucleotide Sok RNA of plasmid R1, which represses synthesis of the toxic Hok protein responsible for postsegregational killing of cells when the R1 plasmid is lost (24). As described in several extensive reviews (14, 114, 116) much was learned about antisense RNA regulation by studies of these RNAs, along with a number of other plasmid and phage antisense RNAs, long before the chromosomally encoded antisense RNAs described here were identified. Given the large numbers of antisense RNAs now being reported to be expressed from chromosomes, these RNAs could have a significant, as yet largely unexplored, impact on bacterial gene expression.

HOW ARE ANTISENSE RNAs BEING FOUND?

Although a few chromosomally encoded antisense RNAs were found by serendipity during the characterization of specific genes, the large increase in reported antisense RNAs has come from genome-wide searches for sRNAs and from transcriptome analysis. As illustrated by examples in **Figure 1**, some of the antisense RNAs are short, around 100 nucleotides in length, similar to the antisense RNAs described for plasmids and bacteriophage. However, other chromosomally encoded antisense RNAs are much longer and in some cases correspond to the 5' or 3' extension of an mRNA transcribed from an adjacent protein-coding gene. For example, in *Listeria monocytogenes*, the long 5' untranslated region (UTR) of the *mogR* transcript overlaps three genes involved in flagellar synthesis encoded on the opposite strand (103). There is also significant variability in the region of overlap between the sense and antisense RNAs. Antisense RNAs can overlap the 5' end, the 3' end, the middle, or the entire gene

encoded opposite. These features have influenced how the antisense RNAs are found.

Prediction by Computational Approaches

The initial searches for chromosomally encoded sRNAs were computational and focused on the identification of conservation and predictions of RNA structure as well as orphan promoter and Rho-independent terminator sequences in intergenic regions (reviewed in 57). By excluding coding regions, these studies generally did not detect RNAs encoded opposite known genes. One exception was a study in which a Gapped Markov Model Index, developed based on properties of known sRNAs, tRNAs and rRNAs, was used to search both intergenic regions and regions antisense to annotated genes to predict novel sRNAs in the model organism *Escherichia coli* (122). This search yielded 133 candidates, of which 46 candidates were predicted antisense to annotated open reading frames (ORFs). Of the five candidates tested, only one, an approximately 350 nucleotide RNA, was detected by Northern analysis. A more recent search for terminator sequences in the entire genome of the cyanobacterium *Synechocystis* sp. PCC6803 led to the report of 73 strong antisense RNA candidates, of which 28, ranging in size from 65 to over 1,000 nucleotides, were detected by Northern analyses and 5'-end mapping (23).

Although most of the initial computational searches for sRNAs focused on intergenic regions, subsequent studies revealed that some of the "intergenic" sRNAs are actually antisense to mRNAs. For example, in *E. coli*, four homologous, approximately 140 nucleotide sRNAs, now denoted Sibs (3, 86, 118), were subsequently found to be transcribed from the DNA strand opposite genes encoding the small toxic Ibs proteins (18). Additionally, the 109 nucleotide *E. coli* GadY sRNA (10) was shown to be encoded opposite the 3' end of the *gadX* mRNA (70). Three RNAs (22–430 nucleotides in length) of the nine sRNAs initially detected in the Gram-positive pathogen *L. monocytogenes*

overlap the ends of mRNAs encoded on the opposite strand (61). Similarly, two RNAs (approximately 60 nucleotides in length) out of six sRNAs detected in the Gram-negative pathogen *Helicobacter pylori* are encoded opposite genes of known function (121). As a final example, in the pathogen *Salmonella enteria*, 11 RNAs (90–450 nucleotides in length) of the 19 sRNAs confirmed to be expressed from the intergenic regions of pathogenicity islands are partially antisense to transcripts from neighboring genes (71). In this last study, Northern and primer extension analyses of RNA taken from strains expressing the antisense RNAs on multicopy plasmids revealed that overexpression of the antisense RNAs had varied effects on the corresponding mRNA; high levels of some antisense RNAs led to decreased levels of the corresponding mRNAs, one antisense RNA appeared to undergo mutual degradation with its target mRNA, and overexpression of yet another antisense RNA resulted in increased levels of the target mRNA.

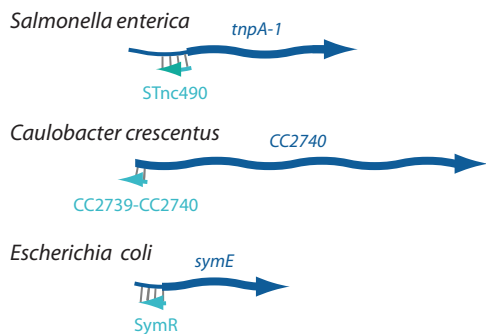
As the properties of an increasing number of antisense RNAs are understood, algorithms to search for this category of sRNA will undoubtedly improve. It is worth considering the possible complication that antisense RNAs, or at least subsets of the antisense RNAs, may have features that are different from other sRNAs and thus might be hard to predict using known sRNAs as training sets. For example, antisense RNAs might not all possess Rho-independent terminators or might fall into more than one class based on their structures and functions.

Direct Detection by Oligonucleotide Microarrays

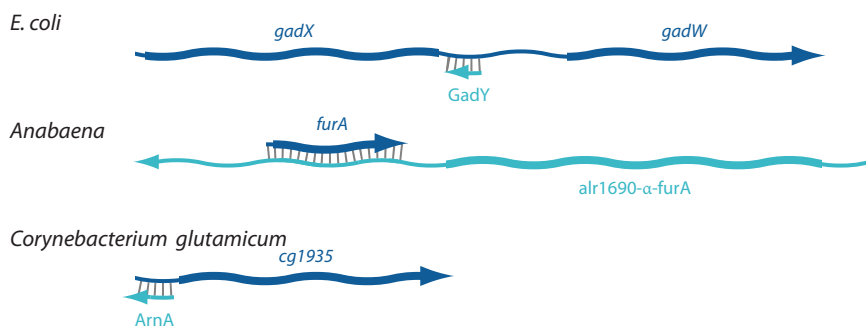
In contrast to the computational approaches focused on intergenic regions, approaches that relied on the direct detection of sRNAs have yielded a higher proportion of antisense RNAs. In fact, antisense expression from over 75% of all annotated genes was observed in one of the first studies of genome-wide expression in *E. coli* using oligonucleotide microarrays with probes for the strand opposite annotated genes (89).

ORF: open reading frame

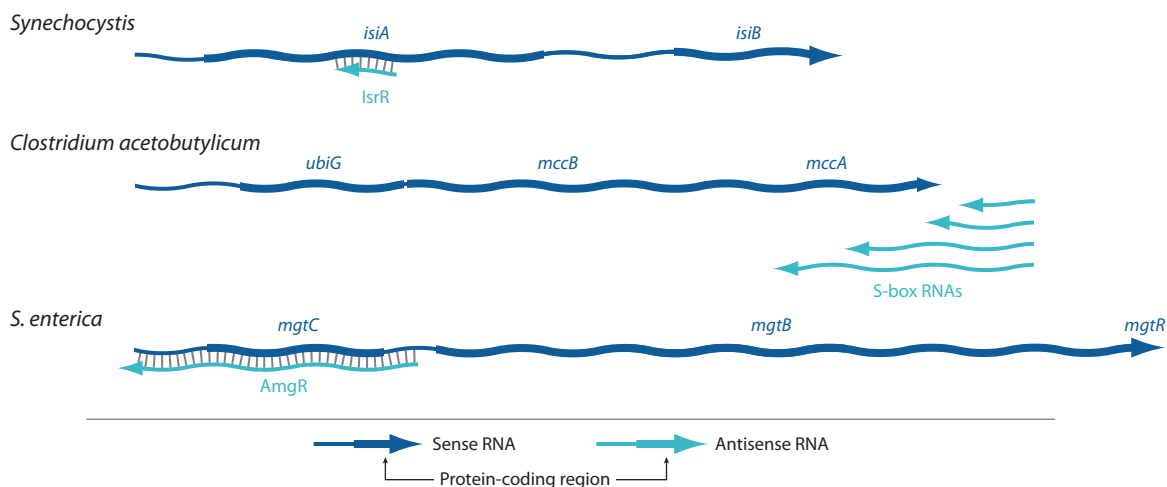
a Transposases and toxic proteins



b Transcription regulators



c Metabolic and virulence proteins



However, no independent verification of these transcripts was carried out, and it is likely that the numbers of antisense RNAs suggested by this study are an overestimate.

Most of the initial oligonucleotide microarrays only had probes for the annotated strand of genes as well as limited coverage of both strands of intergenic regions. Nonetheless, experiments using these arrays resulted in the identification of some antisense transcripts. For example, four RNAs (84–145 nucleotides in length) of 27 sRNAs confirmed by Northern analysis in the freshwater bacterium *Caulobacter crescentus* overlap the 5' or 3' ends of mRNAs encoded on the opposite strand (53), and 24 antisense RNAs (100–600 nucleotides in length) were reported for the cyanobacterium *Prochlorococcus* (98).

The recent trend of using microarrays with oligonucleotides covering both strands of a bacterial genome has led to the identification of many more antisense RNAs. For instance, 127 antisense transcripts ranging in size from 200 to 3,500 nucleotides in length were described for the soil bacterium *Bacillus subtilis* (84). In a similar analysis of the *L. monocytogenes* transcriptome, seven new small antisense RNAs (77 to 294 nucleotides in length) as well as four mRNAs with long 5' UTRs and nine mRNAs with long 3' UTRs that overlap genes encoded on the opposite strand were identified (103).

Possible limitations to the detection of antisense RNAs by oligonucleotide microarrays include artifacts introduced by the production of the cDNAs that are generally used to probe the arrays. For example, some RNAs may be recalcitrant to reverse transcription, resulting in underrepresentation of these transcripts in the final cDNA pool. For other RNAs, replication of the first strand of DNA, especially in the absence of actinomycin D, may lead to false positives (76). These problems can, at least in part, be overcome by hybridizing the RNA directly to the microarrays and detecting hybridization using labeled RNA or antibodies to RNA-DNA hybrids (36), although some RNAs may still be missed by either of these techniques. Another possible complication is cross-hybridization. The oligonucleotides on many microarrays are only 25 nucleotides in length and could hybridize to homologous transcripts. A solution to this problem is the use of microarrays containing longer oligonucleotides and more stringent hybridization conditions.

Direct Detection by Sequencing

Several of the first chromosomally encoded antisense RNAs were found by the sequencing of cDNA clones obtained from various pools of RNA. Such a screen in *E. coli* yielded the 77 nucleotide SymR and the approximately

Figure 1

Length and overlap of selected antisense RNAs with their sense transcripts. (a) RNAs antisense to transposase and toxic protein mRNAs. The 85 nucleotide antisense RNA STnc490 of *Salmonella enterica* completely overlaps the 5' end of the IS200 transposase mRNA *tnpA-1* (97), whereas the 84 nucleotide CC2739-CC2740 antisense RNA of *Caulobacter crescentus* overlaps the IS1111A transposase mRNA *CC2740* by 32 nucleotides at the 5' end (53). In *Escherichia coli*, the 77 nucleotide SymR antisense RNA completely overlaps the 5' end of the mRNA for the SymE toxin (42). (b) RNAs antisense to mRNAs encoding transcription regulators. The 109 nucleotide GadY antisense RNA of *E. coli* overlaps the intergenic region of the dicistronic *gadXW* mRNA, which encodes two transcription regulators (70, 109). The long 2,200 nucleotide *alr1690- α -furA* antisense RNA of *Anabaena* encompasses the entire *alr1690* coding region and extends across the gene encoding the FurA transcriptional regulator into its promoter and regulatory regions (33). The approximately 130 nucleotide ArnA transcript of *Corynebacterium glutamicum* overlaps approximately 99 nucleotides at the 5' end of the *cg1935* mRNA, encoding a putative transcription regulator (123). (c) RNAs antisense to genes encoding metabolic and virulence proteins. The 177 nucleotide IsrR antisense RNA of *Synechocystis* overlaps the central portion of the *isiA* gene, which encodes a protein important under conditions of iron deficiency (13). A series of four antisense S-box RNAs ranging in length from 264 to 1,000 nucleotides in *Clostridium acetobutylicum* are transcribed from a promoter located downstream in the opposite orientation from the *ubiG-mccBA* operon encoding proteins important for S-adenosyl methionine (SAM) recycling. These S-box antisense transcripts can overlap the *mccA* gene by up to 700 nucleotides (2). The 1,200 nucleotide AmgR antisense RNA of *S. enterica* overlaps the entire first gene of the *mgtCBR* operon extending approximately 360 nucleotides into the *mgtC* 5' UTR and promoter region (54).

IVET: in vivo
expression technology

80 nucleotide RyjB RNAs, as well as cDNAs that likely correspond to longer mRNAs that overlap the 3' end of the gene encoded opposite (43). A similar screen in the human pathogen *Mycobacterium tuberculosis* led to the discovery of four antisense RNAs (75–100 nucleotides in length), which, with one exception, are encoded opposite the middle of the sense gene (4).

With the advent of improved sequencing technology, many more antisense RNAs are now being reported. For example, 89 antisense RNAs (12% of all genes) were described for the reduced-genome bacterium *Mycoplasma pneumoniae* (30), 96 antisense RNAs (2% of all genes) were reported for the nitrogen-fixing bacterium *Sinorhizobium meliloti* (88), 27 antisense RNAs (less than 1% of all genes) were reported for *B. subtilis* (37), and 1,005 antisense RNAs (22% of all genes) were reported for *E. coli* (12). It is important to note that the existence of the antisense RNAs was not tested by independent analyses in any of these studies. In other experiments, 25 antisense RNAs (two of which were detected by Northern analysis) were reported for *Chlamydia trachomatis*, which also has a small genome (1), and 127 antisense RNAs (for which four out of nine were detected by Northern analysis) were reported for the pathogen *Vibrio cholerae* (56). Transcription start sites corresponding to an astonishing 969 putative antisense RNAs (46% of all genes) were reported in *H. pylori* (90). Northern analysis supported the expression of 21 of these RNAs (90).

These studies illustrate how antisense RNAs have now been found in a wide range of bacteria, although the percentage of annotated genes that are predicted to have antisense RNAs varies greatly. It should be noted that several of the same problems that plague antisense RNA detection by microarrays, such as the artifacts introduced by cDNA synthesis and amplification, are also problems for antisense RNA detection by sequencing.

Detection of Promoter Elements

Additional evidence of antisense transcription has come from the mapping of promoter

elements through reporter gene fusions, computational promoter predictions or assays of the genome-wide binding of RNA polymerase or other transcription factors. In the plant bacterium *Pseudomonas fluorescens*, in vivo expression technology (IVET) led to the identification of 10 soil-induced transcripts (15–1265 nucleotides in length) encoded opposite annotated ORFs (92). In *E. coli*, promoter elements were predicted antisense to 119 coding sequences (64), and RNA polymerase binding in chromatin immunoprecipitation assays suggested transcription antisense to 25 genes (81). However, no independent analyses to test for the presence of the antisense RNAs were carried out. In another study, promoter activity was detected for a number of chromosomal *lacZ* fusions antisense to annotated genes in *E. coli*, but no transcripts were detected for the eight putative antisense RNAs examined by Northern analysis. This finding suggests that although the chromosome contains numerous promoter sequences, the transcripts may never accumulate (44). As more promoters, transcription start sites, and RNA polymerase binding sites are mapped, it will be useful to determine the extent of overlap in the sets of antisense RNAs predicted by the different approaches. Thus far for *E. coli* and *B. subtilis*, the overlap between sets has been surprisingly limited (12, 37, 44, 64, 81, 84).

Considerations

The prospect of so many antisense RNA regulators is exciting, but some caution is warranted. The expression of only a few of the antisense transcripts has been confirmed by independent means, which is critical given the possible artifacts mentioned above. In addition, some of the Northern blots used to verify antisense RNAs show ambiguous bands. Finally, an even smaller number of antisense RNAs have an identified function. It is possible that some of the transcripts are a result of nonspecific transcription or occasional readthrough from upstream or downstream genes and thus do not have physiological functions. In addition, it is worth

considering whether both the sense and antisense RNAs are expressed in the same cell. Recent genome-wide quantitation of transcript levels suggests that many transcripts are present at levels such that there is less than one RNA molecule per cell (75). Thus, although both sense and antisense transcripts are detected in the pools of cells used for the experiments, they may never actually be present in the same cell. Finally, it is conceivable that a subset of antisense RNAs with true physiological functions are so short lived that they can never be detected.

Further confirmation and characterization of the antisense RNAs are critical before strong conclusions about the total number of antisense RNAs can be drawn. For these studies, it is necessary to eliminate the expression of the antisense RNA to ensure that the observed signal is due to the RNA and to determine whether there are any phenotypes associated with the lack of the RNA. This may require the introduction of mutations that eliminate the antisense RNA promoter but are neutral with respect to the sense gene, or the synthesis of an anti-antisense transcript to neutralize the antisense RNA. Many studies are carried out with overexpression of the antisense RNA. This approach can provide important insights yet also can introduce artifactual effects that are not observed when the antisense RNA is expressed at endogenous levels. Evidence for regulated expression as well as conservation in closely related species can give weight to the conclusion that a specific antisense RNA has a physiological role.

WHAT ARE ANTISENSE RNAs DOING?

Antisense transcripts have been reported opposite genes encoding a wide variety of activities, but very few of these antisense RNAs have been examined for function. However, for those antisense RNAs for which an effect on sense gene expression has been shown, the proteins encoded by the sense genes can be grouped

into some general categories, providing the first functional insights.

Repressing Transposase and Toxic Protein Synthesis

Several antisense RNAs are encoded opposite genes encoding transposases (**Figure 1a**). One of the first antisense RNAs to be discovered, RNA-OUT of the transposon Tn10, was found to repress transposition by reducing transposase levels. Two antisense RNAs discovered in *S. enterica* (71, 97), two in *C. crescentus* (53), and three in *L. monocytogenes* (103) also are encoded opposite transposase genes. Thus, an important function of antisense RNAs in bacteria, as in eukaryotes (reviewed in 60), could be to inhibit transposition.

An increasing number of chromosomally encoded antisense RNAs that downregulate the synthesis of potentially toxic proteins, as was first found for the Sok RNA of plasmid R1, are also being discovered (19; reviewed in 17, 26) (**Figure 1a**). Most of the proteins whose synthesis is repressed by these antisense RNAs are small (less than 50 amino acids), hydrophobic, and toxic at high levels. Although the cellular roles of the proteins are not yet known, it is clear that the synthesis of certain potentially toxic proteins is tightly repressed. For example, the *E. coli* SymE protein is maintained at low levels by the LexA repressor of the SOS response, the SymR antisense RNA, and the Lon protease (42).

The expression of only a few antisense RNAs has been assayed under multiple conditions. However, some of the RNAs antisense to transposase, and toxin genes, such as SymR, appear to be present constitutively in the cell. All of these antisense RNAs have been found to act as repressors, consistent with a role in the continuous tight repression of potentially detrimental proteins.

Regulating the Levels of Transcription Regulators

Other antisense RNAs have been shown to regulate the synthesis of transcription regulators. In *E. coli*, the GadY RNA was

originally identified in a computational search of intergenic regions (10) but was later found to overlap the 3' UTR of the *gadX* mRNA (70) (**Figure 1b**). Both *gadX* and the downstream *gadW* gene encode transcription regulators of the acid stress response, and further studies of the transcripts in the *gadXW* region showed that the two genes are cotranscribed (109). Overexpression of the GadY RNA results in processing of the *gadXW* mRNA giving rise to separate *gadX* and *gadW* transcripts that accumulate to higher levels than the full-length mRNA (70, 109) (J.A. Opdyke, E.M. Fozo, M.R. Hemm & G. Storz, unpublished observations). Removal of the region of *gadX*-GadY base pairing eliminates processing and leads to decreased levels of the *gadX* mRNA, even with GadY overexpression (70). GadY is specifically induced in stationary phase in an σ^S -dependent manner, and the increased levels of the GadX transcription regulator that result from the GadY-directed processing lead to increased expression of GadX target genes (70).

In many bacteria, Fur (ferric uptake regulator) is a key transcription repressor of genes involved in iron uptake and metabolism in the presence of high iron levels. In the cyanobacterium *Anabaena* sp. PCC 7120, a 2,200 nucleotide antisense transcript, detected by Northern analysis carried out with a double-stranded probe, was shown to decrease *furA* expression and translation (33). End mapping revealed that the antisense transcript starts near the promoter of *alr1690* (encoded downstream and on the strand opposite *furA*) and overlaps the entire *furA* mRNA as well as the *furA* promoter (**Figure 1b**). Elimination of the *alr1690*- α -*furA* antisense transcript resulted in increased levels of the FurA protein (33) as well as the FurA-regulated *alr3808* transcript, implicating *alr1690*- α -*furA* in FurA regulation (34). The promoters of both *furA* and *alr1690*- α -*furA* appear to be bound by NtcA, a regulator of nitrogen metabolism genes (58). A number of proteins involved in nitrogen fixation have iron cofactors, explaining the need to coregulate iron acquisition and nitrogen metabolism. However, it is not clear how the *furA* and the

alr1690- α -*furA* transcripts are regulated relative to each other. It is intriguing that regulatory RNAs, in the form of *trans*-encoded sRNAs in many bacteria and iron response elements in the 5' and 3' UTRs of a number of eukaryotic mRNAs, are components of the iron response in many different organisms (reviewed in 62, 85).

A promoter screen in the Gram-positive bacterium *Corynebacterium glutamicum* led to the identification of the approximately 130-nucleotide antisense RNA, ArnA, encoded opposite the 5' end of the *cg1935* gene (123) (**Figure 1b**). This gene encodes a protein that shares similarity with the GntR family of transcription regulators. The ArnA RNA was detected only after cells were incubated at high temperature, suggesting possible regulation by the heat shock sigma factor SigH. The effects of ArnA on *cg1935* were examined using a reporter construct, which gave reduced expression at high temperature in the absence of ArnA, suggesting a potential role for ArnA in stabilizing the mRNA encoding the putative transcription regulator. However, further experiments are needed to clarify the mechanism of action and physiological role of ArnA. The *L. monocytogenes* RliH RNA also overlaps a gene encoding a putative transcription regulator (61), and we predict that still other antisense RNAs will be found to modulate the levels of transcription regulators, either positively (like GadY or ArnA) or negatively (like *alr1690*- α -*furA*).

Modulating the Levels of Metabolic and Virulence Proteins

Antisense RNAs have also been shown to regulate the expression of some metabolic enzymes and virulence factors. One example is the 177 nucleotide IsrR antisense RNA encoded opposite the iron stress-induced IsiA protein in the cyanobacterium *Synechocystis* sp. PCC6803 (13) (**Figure 1c**). The levels of IsiA, a protein that enhances light absorption by forming an antenna ring around photosystem I, are elevated under conditions of iron deficiency and reduced under conditions of iron excess.

Studies of IsiA expression revealed that the response to iron is controlled, at least in part, by the IsrR RNA, which was first detected by Northern analysis of the transcripts in the *isiAB* region. IsrR overexpression leads to decreased levels of IsiA, and depletion of IsrR leads to premature *isiA* expression.

In *Clostridium acetobutylicum*, computational studies predicted an antisense RNA containing a S-adenosyl methionine (SAM)-responsive, S-box riboswitch element convergent to the *ubiG-mccBA* operon, which encodes enzymes involved in the conversion of SAM to cysteine (87). Four S-box containing antisense RNAs of 264–1000 nucleotides in length subsequently were detected by Northern analysis (Figure 1c), and a reduction in MccB protein activity in the presence of sulfur sources was shown to be dependent on the promoter for the S-box antisense RNAs (2). Given that the longer antisense RNAs were not detected in the presence of methionine, this led to the model that, in the absence of SAM, there is readthrough of the S-box riboswitch generating the antisense RNAs, which in turn leads to a reduction of MccB synthesis.

Northern analysis using double-stranded probes led to the detection of the 1,200 nucleotide AmgR RNA encoded opposite the *S. enterica mgtCBR* operon associated with virulence and survival in macrophages (54). AmgR is transcribed from within the *mgtC-mgtB* intergenic region and overlaps the entire *mgtC* gene extending into the *mgtC* 5' UTR (Figure 1c). Expression of both the antisense AmgR RNA and the sense *mgtCBR* mRNA was found to be induced by the response regulator of the PhoQ/PhoP two-component system in response to low Mg^{2+} levels. The presence of AmgR is associated with degradation of the *mgtC* mRNA and a corresponding decrease in MgtC, and to a lesser extent MgtB, protein levels. MgtC protein levels in turn impact *S. enterica* virulence in mice. A *S. enterica* strain overexpressing AmgR was less virulent, whereas an *amgR* promoter mutant strain was more virulent in mice compared to the wild-type control strain.

IsrR, the S-box RNAs, and AmgR illustrate how antisense RNAs might be a factor in modulating the levels of a wide range of metabolic and virulence proteins. Although not well characterized, antisense RNAs are also proposed to impact flagellar synthesis in *H. pylori* (121), *L. monocytogenes* (103), and *S. enterica* (117). In all of these examples, antisense RNAs introduce an additional layer of regulation to systems whose expression is already extensively regulated.

Reasons for Antisense Regulation

One relevant question is why regulation by an antisense RNA might be advantageous over other types of regulation. From looking at the examples described above, two possible answers arise. First, antisense RNAs could provide an advantage when the levels of a particular protein need to be repressed very tightly and expressed under very select circumstances, as in the case of a transposase or toxin. Second, many of the characterized antisense RNA targets are subject to extensive regulation, where the antisense RNAs provide yet one more level of control. For example, the levels of *S. enterica* MgtC are regulated at the transcriptional level by PhoPQ, at the posttranscriptional level by the AmgR antisense RNA, and at the level of protein stability by the MgtR peptide (54). It is interesting to note that for the *mgtC* mRNA-AmgR RNA pair, the same regulator controls expression of both transcripts. The same is also true for other sense-antisense pairs. Although this arrangement at first seems counterintuitive, it may allow for the establishment of desired regulatory circuits.

So far, antisense RNAs appear to fall into two classes with respect to how their transcription is regulated; some appear to be expressed constitutively like the *E. coli* SymR RNA, whereas for others, transcription is tightly controlled. Two additional examples of antisense RNAs that show strong regulation can be found in *B. subtilis*. Here, expression of a 750 nucleotide RNA encoded opposite the 3' end of the *yabE* gene is regulated by the

SAM: S-adenosyl methionine

extracytoplasmic σ factors σ^X and σ^M (15), whereas expression of the 280 nucleotide SurA RNA encoded opposite the 5' end of *yndL* is under the indirect control of Spo0A, the master regulator of sporulation (95). Other trends in the expression and function of antisense RNAs should become evident as more RNAs are characterized. For example, it will be interesting to see how many short and long antisense RNAs have physiological functions and whether the short antisense RNAs have roles that are different from the longer antisense RNAs.

HOW DO ANTISENSE RNAs ACT?

As already mentioned, antisense RNAs can overlap the 5' end, the 3' end, the middle, or the entire gene encoded opposite. It is also possible that although base pairing may take place at one position, the effects of the base pairing may be propagated to another region of the sense RNA. The sense and antisense RNAs also show a range of expression patterns. In some cases, both transcripts show similar patterns of expression: both high or both low under a specific condition. In other cases, the transcripts show opposing patterns of expression, with the level of one RNA high and the other low under a specific condition. All of these features affect how the antisense RNA might impact the transcription, stability, or translation of the sense RNA.

Transcription Interference and Attenuation

Antisense RNAs can alter transcription of the genes encoded on the opposite strand in two different ways: transcription interference or transcription attenuation (Figure 2a,b). Transcription interference occurs when transcription from one promoter is suppressed by a second promoter present in *cis* (reviewed in 91). This effect was studied for two convergent bacteriophage 186 promoters that produce transcripts overlapping by 62 base pairs at their 5' ends (7). In this study, a stronger promoter reduced the activity of the weaker promoter by 5.6-fold. When the promoters were oriented

divergently, but the transcripts still maintained their regions of complementarity, transcription of the weaker promoter was not affected by transcription from the stronger promoter. In addition, the introduction of a terminator before the convergent weak promoter resulted in reduced interference. These results led to the conclusion that the convergent orientation of the promoters was the source of the interference rather than base pairing.

Transcription interference has been proposed to be the mechanism by which the *S*-box antisense RNAs of *C. acetobutylicum* block expression of the *ubiG-mccBA* mRNA (2). The antisense RNAs generated from the promoter of the *S*-box RNA can overlap the *ubiG-mccBA* mRNA by up to 700 base pairs, but the levels of the *ubiG-mccBA* mRNA were unchanged in a number of ribonuclease mutants, suggesting the antisense RNAs do not impact mRNA processing. Based on this observation and the finding that the antisense RNA provided in *trans* did not affect *ubiG-mccBA* mRNA levels, transcription interference was inferred, but this has not been tested directly.

Antisense RNAs also can alter target mRNA transcription by inducing attenuation, whereby transcription of the opposite strand is prematurely terminated. In a few cases, generally found on plasmids, base pairing of the antisense RNA to the mRNA has been shown to induce the formation of a terminator structure in the target mRNA. For example, the 427 nucleotide RNA β , encoded opposite the *fatDCBA-angRT* iron transport-biosynthesis operon on the virulence plasmid pJM1 in the fish pathogen *Vibrio anguillarum*, was reported to be required for transcription attenuation (99). In vitro transcription and S1 nuclease protection assays indicated that the *fatDCBA-angRT* transcript is terminated after interaction of a stem loop in the *fatA-angR* intergenic region with stem loops of RNA β . Transcription termination results in increased levels of the *fatA* portion of the mRNA compared to the downstream *angRT* portion providing a mechanism for discoordinate expression within an operon. How frequently transcription interference or

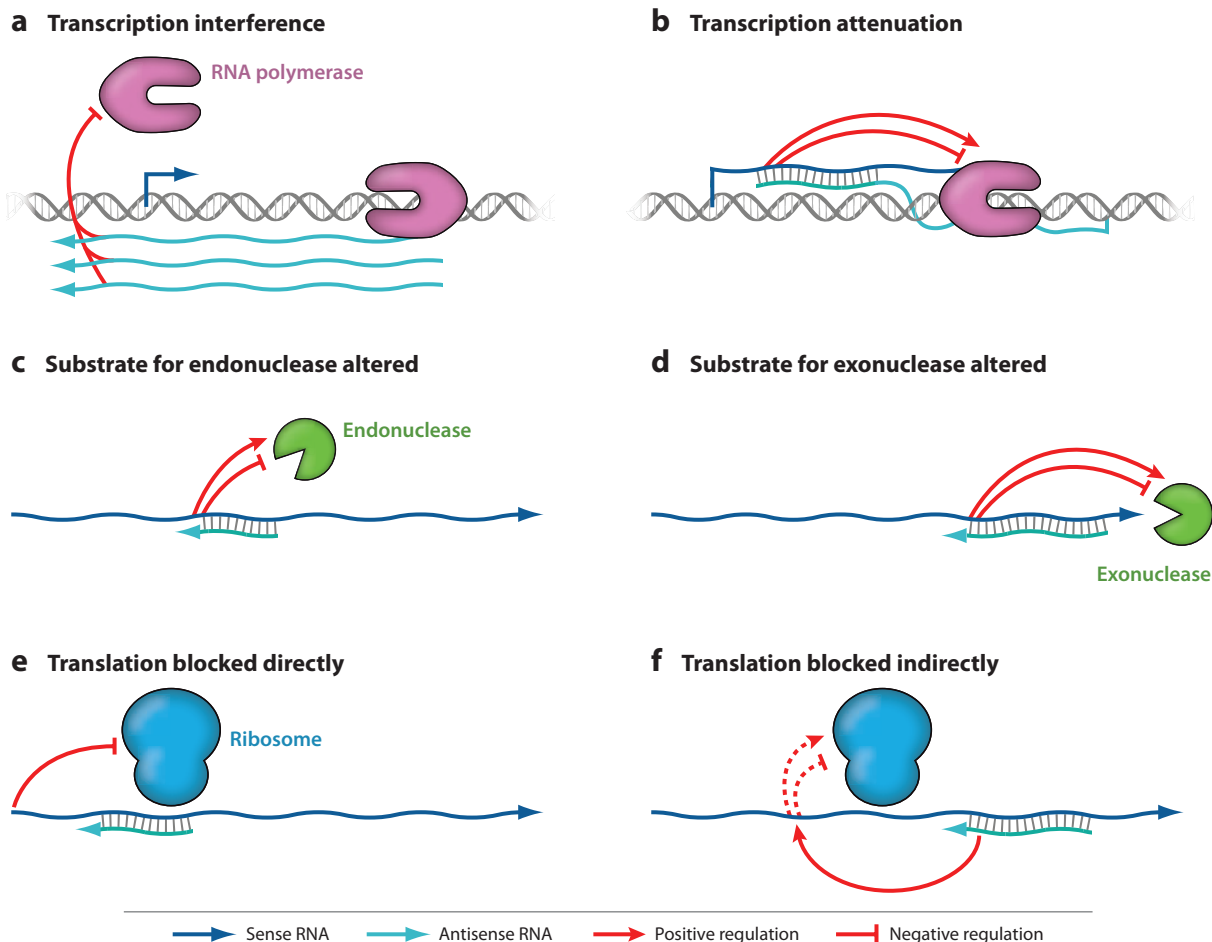


Figure 2

Mechanisms by which antisense RNAs act. (a) Antisense RNAs can induce transcription interference, where transcription from one promoter blocks transcription from a second promoter by preventing RNA polymerase from either binding or extending a transcript encoded on the opposite strand. Transcription interference does not involve base pairing and does not occur when the antisense RNA is provided in *trans*. (b) In transcription attenuation, base pairing of the antisense RNA to the target RNA causes changes in the target RNA structure ultimately affecting transcription termination. Antisense RNAs can also affect target RNA degradation by (c) endonucleases and (d) exonucleases. In these cases, base pairing between the sense and antisense RNAs can either directly generate or block a ribonuclease target site. Antisense RNAs can also indirectly affect the binding of the ribonuclease at a distance from the site of base pairing. Finally, antisense RNAs can (e) directly block ribosome binding or (f) indirectly positively or negatively impact ribosome binding by affecting the target mRNA structure.

attenuation mechanisms involve antisense RNAs remains to be determined.

RNA Cleavage

Antisense RNAs can also impact the stability of a target RNA by either promoting or blocking

cleavage by endoribonucleases or exoribonucleases (**Figure 2c,d**). In many bacteria, two major endoribonucleases have been linked to antisense RNA-induced target mRNA cleavage. The first, RNase III, cleaves double-stranded RNA and is generally associated with rRNA processing (reviewed in 8). Its ability to

RNase III: endoribonuclease that cleaves double-stranded RNA; generally involved in ribosomal RNA processing

RNase E:

endoribonuclease that cleaves single-stranded RNA; part of the degradosome

specifically degrade double-stranded RNA complexes makes it an ideal candidate for processing antisense RNAs base paired with their targets. In fact, RNase III was found to be responsible for cleavage of the *copT-copA* and *hok-sok* plasmid-based sense-antisense RNA pairs in early studies (5, 25). Similarly, RNase III was shown to cleave two sRNAs, RyeA (also called SraC) and RyeB, encoded opposite each other in *E. coli* (113). The antisense RNA-directed cleavage could generate two target RNA halves that are both less stable, both more stable, or differentially stabilized relative to the original target transcript. The second endonuclease reported to be involved in antisense RNA-directed processing is RNase E, which cleaves single-stranded RNA. RNase E is a component of the degradosome, a multiprotein complex that globally affects mRNA stability and has been shown to interact with Hfq (reviewed in 8). The AmgR RNA of *S. enterica* was reported to induce degradation of the *mgtC* mRNA in a manner that requires RNase E but not RNase III (54). Exactly how a double-stranded region of RNA might stimulate RNase E activity is not yet clear, although it is conceivable that the 5' end of an antisense RNA could provide a monophosphate, which has been shown to stimulate RNase E activity (9). It is also possible that base pairing might block an RNase E recognition site, thus leading to increased stability of the target RNA.

RNase III and RNase E likely are not the only endonucleases involved in antisense RNA-directed processing. Although RNase III was responsible for some of the OOP RNA-directed cleavage of the *cII-O* mRNA of bacteriophage λ , processing was still observed within the regions of base pairing, albeit with a slightly different pattern, in a strain lacking RNase III (52). However, determining what endonucleases are responsible for a particular cleavage event can be difficult due to redundancies between cellular RNases. The fact that several of these enzymes are essential and show autoregulation and cross-regulation also complicates this analysis.

In addition to providing substrates for endonucleases, the base pairing between a sense and an antisense RNA might impact the ability of an exonuclease to degrade a particular target RNA. For example, the region of base pairing might block an exonuclease similar to how stem loops within a transcript can block digestion. Additionally, the base pairing could alter the target RNA structure so that the ends are more or less susceptible to cleavage. Given that only a few studies have been carried out to examine the roles of exonucleases in sRNA and antisense RNA-directed processing, this is an important direction for future studies.

Translation Block

Many *trans*-encoded sRNAs base pair with the Shine-Dalgarno sequences of their target mRNAs, thereby preventing ribosome binding and protein translation (reviewed in 72, 119). Antisense RNAs for which complementarity extends into the 5' UTR of the mRNA target may act by a similar mechanism (**Figure 2e**). The SymR antisense RNA of *E. coli* overlaps the 5' end of the *symE* mRNA (42). In a strain with a *symR* promoter mutation, the levels of the *symE* mRNA were increased threefold, whereas the levels of the SymE protein were increased tenfold, suggesting some of the repression by SymR occurs by blocking translation.

Similarly, the *alr1690- α -furA* antisense RNA overlaps the length of the *furA* transcript including the Shine-Dalgarno sequence. Elimination of the *alr1690- α -furA* transcript resulted in increased levels of the FurA protein consistent with a possible block in translation (33), but further experiments are needed to verify this. Ribosome binding and mRNA stability are often linked, presenting a complication in evaluating whether the primary effect of sense-antisense pairing is a block in translation. For example, the AmgR RNA overlaps the Shine-Dalgarno sequence of its target mRNA *mgtC*, yet has been proposed to destabilize the *mgtC* mRNA in an RNase E-dependent manner (54). Base pairing between the sense and antisense

RNA also could positively or negatively impact translation by altering the structure of the target RNA at a ribosome binding site distant from the region of base pairing (**Figure 2f**).

Dual Functions

Modulating the transcription, stability, or translation of a *cis*-encoded gene might not be the only function of a particular antisense RNA. One of the short antisense RNAs identified in *P. fluorescens* was reported to encode a small protein (93), and some of the longer antisense RNAs, such as *alr1690- α -furA*, are known to encode proteins. Subsets of these RNAs may have dual roles as mRNAs and base-pairing RNAs, although others might function solely as mRNAs. Antisense RNAs also might act as both *cis*- and *trans*-encoded base-pairing RNAs. Among the shorter antisense RNAs, the *E. coli* GadY RNA has been shown to bind to Hfq, the RNA chaperone required by *trans*-encoded base-pairing sRNAs but thus far not thought to be required by *cis*-encoded base-pairing RNAs. This suggests GadY might base pair with other mRNA targets (70). Similarly, the RliE RNA of *L. monocytogenes* (61) and the ASdes and ASpks RNAs of *M. tuberculosis* (4) have been predicted to base pair with a number of targets other than the transcript encoded opposite the antisense RNA gene. Further investigation is required to determine whether many antisense RNAs have dual roles.

HOW DO ANTISENSE RNAs BASE PAIR?

The mechanisms of base pairing between an antisense RNA and its target have been studied most extensively for the RNAs found encoded on plasmids and transposons using *in vitro* structure probing and mobility shift experiments. Two general mechanisms for how base pairing proceeds have been proposed. In the first mechanism, denoted a one-step mechanism, an initial point of contact between the antisense RNA and the mRNA target leads to complete duplex formation. In the second

mechanism, denoted a multi-step mechanism, an initial transient interaction between the antisense RNA and the mRNA target requires stabilization by a protein or the formation of additional base pairs. The stable complex then can go on to form a complete duplex between the antisense RNA and the mRNA target, although this proceeds slowly and, in cases where it has been examined, does not appear to be required for regulation. In many cases of base pairing, stem loops containing a YUNR U-turn motif [a motif containing a pyrimidine (Y) followed by a uracil (U), any nucleotide (N), and a purine (R)] are important for the RNA-RNA interactions (reviewed in 20).

One-Step Mechanism

The base pairing between the Hok and Sok RNAs of plasmid R1 proceeds via a one-step binding mechanism, whereby a single-stranded 5' region of the antisense Sok RNA base pairs with a stem loop containing a YUNR U-turn motif in a processed version of the *bok* mRNA (21, 22, 101). Initial base pairing then progresses to more complete duplex formation between the Sok RNA and the *bok* mRNA, preventing Hok protein translation. The base pairing between the transposase mRNA and RNA-OUT of the transposon Tn10 similarly involves one-step base pairing. The YUNR U-turn loop of RNA-OUT and the 5' region of the transposase mRNA first interact because mutations in either of these regions reduce base pairing (47). Again, a more complete duplex is subsequently formed.

Multi-Step Mechanism

Base pairing between RNAII and RNAI of plasmid ColE1 and the *repA* mRNA and CopA of plasmid R1 proceeds via a multi-step mechanism. The first step of base pairing between RNAI and RNAII is the formation of a transient kissing complex through the single-stranded regions of RNAI and RNAII stem loops that both contain YUNR motifs (104). In the second step, the RNAI modulator protein, Rom (also named

YUNR: a motif containing a pyrimidine (Y) followed by a uracil (U), any nucleotide (N), and a purine (R)

Kissing complex: initial base-pairing interaction between an antisense RNA and its mRNA target

TRD: target recognition domain

Rop), recognizes the structure of the kissing complex (rather than the nucleotide sequence) and stabilizes this RNAI-RNAII complex (82, 105, 108). The stabilized interaction between the loop structures then brings the 5' end of RNAI in close proximity to its complementary region in the RNAII transcript, facilitating more complete duplex formation (104, 105).

The first step in the interaction between the CopA antisense RNA and the CopT target region in the *repA* mRNA leader sequence also involves the formation of a kissing complex. This requires stem loop II of CopA contacting the YUNR motif in the CopT stem loop II (22, 35, 49, 50, 78–80). The formation of an extended kissing complex involving nucleotides other than the initial stem loop II region is required to stabilize the initial kissing complex. After formation of the extended kissing complex, the base pairing progresses to a structure in which two helices are formed between CopA and CopT. This is further stabilized by a third helix generated by base pairing between a stretch of single-stranded nucleotides in the 5' end of CopA with a complementary region in CopT (48, 49, 59). This stabilized structure is sufficient for replication control, as formation of a completely base-paired duplex is very slow in vitro and is unnecessary for replication control in vivo (59, 79, 115).

Although the sense-antisense base-pairing interactions for the transposon and plasmid RNAs have been characterized extensively, much less is known about chromosomal sense-antisense base pairing. However, a recent study of the interactions between the chromosomally encoded *ibs* mRNAs and Sib antisense RNAs suggests that base pairing may proceed by similar mechanisms (31). Structure probing of the interactions between the Sib and *ibs* RNAs revealed that base pairing is initiated via two regions. A YUNR U-turn motif, located in one target recognition domain (TRD1) of the SibC RNA, recognizes a complementary YUNR U-turn motif in the *ibsC* mRNA. A second domain (TRD2) within a single-stranded region between the adjacent stems of SibC could also initiate base pairing with the *ibsC* mRNA. The

interactions between both of these target regions lead to more complete complex formation but do not result in formation of a fully base-paired duplex. Mutant versions of SibC showed that either TRD1 (opposite the *ibsC* ORF) or TRD2 (opposite the *ibsC* translation initiation region) were able to repress IbsC protein synthesis independently, perhaps through different mechanisms, again indicating complete duplex formation may not be required to prevent IbsC toxicity. Although the TRD1-mediated interaction involved a YUNR U-turn motif, the TRD2 region is similar to the single-stranded regions of Sok and RNA-OUT that are involved in pairing. Whether other chromosomally encoded antisense RNAs use the loop-loop YUNR interactions, a loop–single strand interaction, a combination of both, or completely different base-pairing interactions remains to be determined. It will also be of interest to test whether the effects are reversible, given that it is conceivable that RNA helicases may dissociate base-paired RNAs.

Additional factors, which are likely to influence antisense RNA–target RNA base pairing and have not been examined in detail, include the relative amounts of the two transcripts as well as other proteins interacting with the transcripts. The amounts of both the sense and antisense RNAs have been determined in very few cases. For the *symE*–SymR pair in *E. coli*, the amounts of the SymR antisense RNA are in significant excess (tenfold higher) over the *symE* mRNA (42). In addition, the structures of the RNAs and the availability of different regions for base pairing may vary, as the RNAs are being transcribed or processed. Finally, portions of one or both of the RNAs may be translated and bound by ribosomes, which undoubtedly will also impact the ability of the RNAs to base pair.

CAN ANTISENSE RNAS BE EXPLOITED?

Not surprisingly, the use of synthetic antisense RNAs as antibiotics and in biotechnology has been under investigation for quite some

time. Two general approaches for delivering synthetic antisense transcripts have been pursued: expression of an antisense transcript from a gene introduced into the cell and the direct delivery of antisense oligonucleotides. Degradation is a problem with both in vivo synthesized RNAs and the direct delivery of oligonucleotides. To circumvent this problem, antisense RNAs that form more stable hairpin structures using paired termini have been used (67). In addition, antisense oligonucleotides have been modified by adding polyamide backbones (PNAs), substituting morpholine rings for the deoxyribose rings (PMOs), and replacing the internucleoside linkages with phosphorothioates (PS-ODNs) (reviewed in 83). However, the cell walls of bacteria can inhibit uptake of antisense oligonucleotides, PNAs, and PMOs, limiting their applicability. To address this problem, cell-permeable cationic peptides have been conjugated to PNAs and PMOs, increasing cellular uptake and potency and extending retention of the peptide-PNA within the cell (28, 69).

Synthetic antisense RNAs have already been used in a number of applications. They have been shown to inhibit growth of *E. coli* (28, 63, 69, 102), *S. enterica* (65), *Staphylococcus aureus* (68), and *M. tuberculosis* (32) when targeted to essential genes. Because antisense RNAs allow for the conditional repression of target genes, they also have been employed to study bacterial growth and metabolism (6, 27, 41, 77, 111) and to characterize known or putative virulence factors (39, 66). Antisense RNAs have been used to sensitize bacteria to antibiotics (38, 40, 45), identify novel antibiotics (74, 124), identify antibiotic targets (29, 32, 40), or clarify the mechanisms of action of potential new drugs (40). In addition, antisense technology has allowed the induction of bacterial suicide without the need for antimicrobial compounds. For example, in *E. coli*, introduction of a PNA complementary to the Sok antisense RNA resulted in the synthesis of the toxic Hok protein and cell death (16). Finally, antisense RNA technology has been used to alter bacterial gene expression in industrial processes in order to

produce chemicals more efficiently with fewer unwanted byproducts (11, 94, 110, 112) and to optimize protein production (46). *C. acetobutylicum*, an important industrial bacteria, is able to ferment a number of complex carbohydrate sources, like cellulose, into important industrial solvents, acids, and biofuels such as ethanol, acetone and butanol (reviewed in 73). Genetic manipulations of *Clostridium* are difficult, but antisense technology has been successful in modifying *C. acetobutylicum* primary metabolism to produce the desired industrial compounds and biofuels (11, 94, 110, 112).

Despite the successes of antisense RNA technology, a number of issues still need to be addressed. In general, synthetic antisense RNAs are targeted to the Shine-Dalgarno sequence; however, different regions of specific target mRNAs may correlate with more efficient repression. Additionally, antisense oligonucleotides, due to their short length, can have off-target effects. The use of multiple oligonucleotides against the target RNA, which should not all have the same off-target effects, can be a control for this problem. Undoubtedly, a greater understanding of endogenous target RNA-antisense RNA base pairing and target inhibition will facilitate the engineering of better synthetic antisense RNAs for clinical, industrial, or laboratory use.

PERSPECTIVES

The study of antisense RNAs is one of the most exciting areas of regulatory RNA research in bacteria given the many questions that remain to be answered. With the recent advances in transcriptome analyses, hundreds of antisense RNAs are being reported. How many of these newly reported antisense RNAs have physiological functions? Will most have dual functions as mRNAs and base-pairing RNAs or as base-pairing RNAs that act on both *cis*- and *trans*-encoded targets? Many of the characterized antisense RNAs repress transposon and toxic protein synthesis, and modulate the expression of genes encoding transcription regulators or other genes that show complex regulation. Will

the targets of newly identified functional antisense RNAs fall into these same categories? Known antisense RNAs can act through a number of mechanisms, including transcription interference and attenuation, RNA stability, and ribosome binding. Will one or another mechanism predominate as more RNAs are characterized? The length of antisense RNAs can vary from tens to a few thousand nucleotides. Will antisense RNAs of different lengths be

associated with different mechanisms of action? Studies of short antisense RNAs have revealed that base pairing with the target RNA generally is mediated by loop-loop interactions. Will longer antisense RNAs base pair using the same mechanisms? With more and more tools available for the identification of chromosomally encoded antisense RNAs and increasing interest in antisense RNAs, answers to these questions should be forthcoming.

SUMMARY POINTS

1. Bacterial chromosomes encode a number of regulatory RNAs, many of which act by base pairing. Base-pairing RNAs fall in to two categories. *Trans*-encoded sRNAs are encoded at genomic locations distinct from their target mRNA(s) and act via limited base pairing. *Cis*-encoded, or antisense RNAs, are encoded on the DNA strand opposite other genes and thus can act via more extensive base pairing.
2. With recent advances in direct detection methods, the numbers of antisense RNAs reported have increased dramatically from just a few to hundreds. However, validation and functional characterization for most of these newly reported antisense RNAs are lacking.
3. The first antisense RNAs to be discovered (associated with plasmids, phage, and transposons) were small, generally only a few hundred nucleotides in length. However, several of the newly described, chromosomally encoded antisense RNAs are reported to be larger than 1,000 nucleotides in length, in some cases overlapping entire genes and themselves encoding proteins.
4. Antisense RNAs have been shown to repress target mRNAs encoding proteins, such as transposons and toxic proteins, that have the potential to be detrimental to the cell. They also have been shown to positively and negatively impact the expression of transcription regulators as well as a number of other metabolic and virulence proteins, many of which are regulated extensively at other levels.
5. Antisense RNAs can act by altering their target gene transcription through interference or transcription attenuation. Antisense RNAs can also provide or block recognition sites for endonucleases as well as exonucleases. In addition, some antisense RNAs can act like *trans*-encoded sRNAs to modulate ribosome binding and translation. Finally, antisense RNAs can have dual functions and act as both mRNAs and antisense RNAs or both *cis*- and *trans*-encoded RNAs.
6. Antisense RNAs can initiate base pairing via stem loops containing YUNR U-turn motifs or via single-stranded regions of sequences complementary to regions on their target mRNAs. Base pairing can proceed either by a one-step mechanism whereby an initial contact proceeds to more complete duplex formation or by a two-step mechanism whereby the initial kissing complex is first stabilized by a protein or additional contacts before proceeding to more complete duplex formation.

FUTURE ISSUES

1. The hundreds of antisense RNAs now being reported need to be further validated and functionally characterized before generalizations regarding the extent of antisense transcription in bacterial genomes can be made.
2. Future studies should clarify whether antisense RNAs fill a specific regulatory niche in the cell.
3. Much remains to be learned about the mechanisms of antisense RNA action and base pairing, particularly for longer antisense RNAs.

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Errata

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